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Cells from Within: DNA in Life and Death

In the previous chapters, we have discussed how it is possible to stain proteins on the surface and inside of cells and then to analyze these cells for the presence and intensity of that stain. In addition to protein, another biochemical component that can be used to classify different types of cells is, of course, DNA. It should therefore come as no surprise that flow cytometrists have developed methods for analyzing DNA content.

FLUOROCHROMES FOR DNA ANALYSIS

By comparison with the fluorochromes used for conjugation to antibodies for staining the proteins of cells, DNA-specific fluorochromes have important differences. In particular, whereas fluorescein, phycoerythrin (PE), and others are fluorescent whether or not they are bound to cells, the DNA fluorochromes fluoresce significantly only when they are bound to their target molecules. In addition, unlike the tight binding of antibody to antigen, DNA fluorochromes are generally in loose equilibrium between their bound and free states. Therefore procedures for analyzing the DNA content of cells involve sending cells through the flow cytometer without washing them to remove the “unbound” fluorochrome. The unbound fluorochrome will not add to background fluorescence because it is hardly fluorescent unless bound to nucleic acid. And washing would, in any case, lower overall specific fluorescence by removing much of the fluorochrome (both bound and unbound) from the cell.

TABLE 8.1. Characteristics of Some Nucleic Acid Stains

Stains	Absorption (nm)	Fluorescence (nm)	Specificities
Hoechst 33342 and Hoechst 33258	346	460	DNA with AT preference; Hoechst 33342 enters viable cells well, Hoechst 33258 less well
DAPI	359	461	DNA with AT preference; slightly permeant to viable cells
Chromomycin A3	445	575	DNA with GC preference; impermeant
Acridine orange	460 (RNA) 480 (DNA)	650 (RNA) 520 (DNA)	DNA and RNA; metachromatic; permeant to viable cells
Thiazole orange	509	525	DNA and RNA; permeant
Ethidium bromide	510	595	Double-stranded nucleic acids; impermeant
Propidium iodide	536 (also UV)	623	Double-stranded nucleic acids; impermeant
7-Amino-actinomycin D (7-AAD)	555	655	DNA and RNA; GC preference; impermeant to viable cells
TO-PRO, TO-TO, PO-PO, PO-PRO, YO-YO, and YO-PRO series	434–747	456–770	DNA and RNA; impermeant
SYTO series	488–621	509–634	DNA and RNA; permeant

Several types of fluorescent stain are available for the analysis of DNA; their characteristics make them suitable for different applications (Table 8.1). The most specific stains (e.g., DAPI and the Hoechst dyes, which stain specifically for AT groups on DNA) require the use of a laser with significant ultraviolet (UV) output. Hoechst dyes as well as a newly developed far-red dye called DRAQ5 (alone of all the current DNA-specific stains) also penetrate the outer

membrane of living cells and can therefore be used for staining living cells with different DNA content for subsequent sorting for separate culture or functional analysis. Chromomycin A3 is specific for the GC bases in DNA and therefore is an appropriate stain for use in conjunction with Hoechst 33258, as will become evident in the discussion of chromosome techniques. Propidium iodide, although not very specific (it stains all double-stranded regions of both DNA and RNA by intercalating between the stacked bases of the double helix) and not able to penetrate an intact cell membrane, has the decided advantage of absorbing 488 nm light and then fluorescing at wavelengths above 570 nm. This means that, in the presence of RNase, propidium iodide can be used as a DNA stain in cytometers with low-power argon lasers. Propidium iodide has therefore become the most common DNA fluorochrome for flow analysis.

More recently, a series of nucleic acid probes has been developed by Molecular Probes (Eugene, OR); these probes have an array of unlikely names (like TO-PRO, YO-YO, and PO-PRO, sounding something like the three little maids from school in "The Mikado") and also provide a large choice of absorption, emission, and nucleic acid binding properties. Other fluorochromes that absorb 488 nm light include acridine orange, which is metachromatic; that is, it fluoresces red if bound to nonhelical nucleic acid (e.g., RNA or denatured DNA) and fluoresces green if bound to helical nucleic acid (e.g., native DNA). Acridine orange has been used effectively by Darzynkiewicz and coworkers to follow the changes in RNA content and in DNA denaturability that occur during the cell cycle. Moreover, the monoclonal antibody against bromodeoxyuridine (a thymidine analog) can be conjugated to fluorescein, and it will then stain DNA that has incorporated bromodeoxyuridine when cells have been pulse-fed with this compound during DNA synthesis. Before discussing the uses of these stains for chromosome and cell cycle analysis, we should first consider the most obvious use of DNA fluorochromes: staining cells for their total DNA content.

PLOIDY

The amount of DNA in the nucleus of a cell (called the 2C or *diploid* amount of DNA) is specific to the type of organism in question.

Different species have different amounts of DNA in their cells (e.g., human cells contain about 6 pg of DNA per nucleus; chicken cells, about 2.5 pg of DNA per nucleus; corn [*Zea mays*] nuclei, about 15 pg; and *Escherichia coli*, between 0.01 and 0.02 pg each). However, within the animal kingdom, with three major exceptions, all healthy cells in a given organism contain the same amount of DNA. The three major exceptions are, first, cells that have undergone meiosis in preparation for sexual reproduction and therefore contain the 1C or haploid amount of DNA typical of a gamete; second, cells that are carrying out DNA synthesis in preparation for cell division (mitosis) and therefore for a short period contain between the 2C amount of DNA and twice that amount; and third, cells that are undergoing apoptosis and have begun to lose pieces of fragmented DNA. (There are other less common exceptions as well: For example, liver cells exist as normal tetraploids, and multiploidy is the rule rather than the exception in plant cells.) Because healthy, normal animal cells from a given individual, with these three major and other minor exceptions, contain the same amount of DNA, measurement of the DNA content of cells can be used to identify certain forms of abnormality. More specifically, the type of abnormality commonly termed *malignancy* is often associated with genetic changes, and these genetic changes may sometimes be reflected in changes in total DNA content of the malignant cell.

It is possible to permeabilize the outer membrane of normal cells (with detergent or alcohol) in order to allow propidium iodide to enter the nuclei. If we then treat the normal cells with RNase in order to ensure that any fluorescence results from their DNA content (without a contribution from double-stranded RNA), we find that the nuclei fluoresce red with an intensity that is more or less proportional to their DNA content. By the use of a red filter and a linear amplifier on the photomultiplier tube, we can detect that red fluorescence. The channel number of the fluorescence intensity will be proportional to the DNA content of the cells. The method is simple and takes about 10 minutes. Flow cytometric analysis of the red fluorescence from the particles in this preparation of nuclei from normal, nondividing cells will result in a histogram with a single, narrow peak (see the first histogram in Fig. 8.1); all the particles emit very nearly the same amount of red fluorescence. This supports our knowledge that all

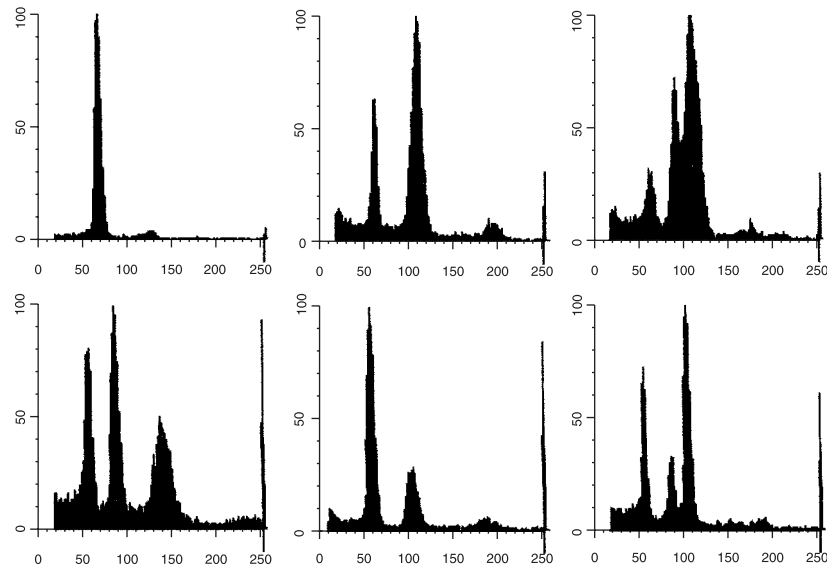


Fig. 8.1. Propidium iodide fluorescence histograms from nuclei of cells aspirated from normal tissue (upper left) and malignant breast tumors. Data courtesy of Colm Hennessy.

normal, nondividing nuclei from any one organism contain the same amount of DNA.

If we then look at a preparation of material from malignant tissue, we find that the fluorescence histogram often indicates the presence of cells with the “wrong” amount of DNA, as well as cells with the amount of DNA that is normal for the organism in question. The normal cells are said to be *euploid* or normal diploid, and the abnormal cells are termed *aneuploid* or *DNA aneuploid* (flow cytometrists have hijacked these terms from cytologists and use them to refer to total DNA content of cells; cytologists feel that the use of the euploid/aneuploid classification is ambiguous unless chromosomes have been counted). Histograms from examples of some malignant tissues are shown in Figure 8.1. The abnormal peak or peaks may have more or less DNA than normal cells (hyperdiploid or hypodiploid). Because our basic axiom is that all normal cells from an organism contain the same amount of DNA, any tissue that yields a DNA flow histogram

with more than one peak contains, by definition, abnormal cells. Flow cytometry is therefore a quick and straightforward method for measuring the particular type of pathology that results in cells with abnormal DNA content.

In the 1980s, at the same time that scientists were beginning to realize that changes in the total DNA per nucleus could be measured by flow cytometry and that this could be an indicator of the presence of abnormal tissue, David Hedley in Australia discovered that when fixed tumors embedded in paraffin blocks were de-waxed and rehydrated, released nuclei could be analyzed by flow cytometry for DNA content. Although the absolute fluorescence intensity of propidium iodide-stained nuclei released from fixed material was lower than that from fresh material, the patterns revealed in the flow histograms were similar. The finding that material from paraffin blocks could be used to analyze DNA content (ploidy) of the individual cells had two important consequences. A very large amount of archival clinical material was suddenly amenable to DNA analysis, and because some of the archival material was 5, 10, and 20 years old, long-term clinical follow up of the patients was immediately available.

The correlation of DNA flow histograms with prognosis became a quick and simple proposition. As a result of Hedley's technique, the corridors in hospitals all over the world were suddenly filled with swarms of young clinicians beating paths to the doors of their pathology departments (and then on to the flow cytometry facilities). An enormous number of publications emerged from the use of this technique on many different types of human material. Aware of the risk of overgeneralization and without the time or space in this book for a full discussion of clinical correlations, I can probably safely say here that most (but not all) of the published results showed a correlation between abnormal flow histograms (aneuploidy) and unfavorable prognosis. Furthermore, many of the publications also showed that flow histograms provide information about prognosis over and above that provided by other, more traditional prognostic indicators.

Although the use of DNA flow histograms to diagnose aneuploidy is both easy and rapid, it does have certain drawbacks that should be made clear. The first drawback results from the nature of malignant changes themselves: Not all malignancies will result from DNA changes that are detectable by a flow cytometer. Current knowledge of the causes of malignancy is far from perfect. Nevertheless, it is

possible to imagine that some malignancies may result from changes that are not related to DNA, and other malignant changes may affect a cell's DNA but not in a way that could ever be detected in a flow histogram of propidium iodide fluorescence. For example, chromosome translocations may lead to gross abnormalities in genetic coding, but do not lead to any change at all in the total DNA content of a nucleus. Translocations can be detected easily by microscopic analysis of the individual banded chromosomes in a mitotic spread, but translocations will never be detected by flow cytometry of nuclei stained with propidium iodide. Whereas extra copies (e.g., trisomy) of a large chromosome may result in a measurable shift in the total DNA content of a nucleus, trisomy of a small chromosome may not be detectable in this type of flow analysis (a large chromosome might contain 4% of a cell's total DNA, but a small chromosome has less than 1%). Similarly, small insertions or deletions to chromosomes may lead to changes in DNA content that are too small to be detected by flow cytometry. Any change resulting in less than 3–5% difference in total DNA content may be difficult to detect by flow cytometry, although, to a geneticist, a 3% deletion or insertion involves a large number of base pairs with a potentially enormous amount of misplaced genetic information.

While this first kind of difficulty is an intrinsic limitation of the DNA flow histogram technique, a second problem is more in the nature of a continuing question about interpretation. Although Figure 8.1 shows examples of histograms that provide undoubted evidence of abnormality, Figure 8.2 shows another series of histograms that are considerably more difficult to interpret because of problems arising with so-called wide coefficient of variation (CV) data. The real question concerns our ability to rule out the existence of near-diploid abnormalities when the width (CV) of a peak is very broad. In theory, because all normal nuclei contain the same amount of DNA, the peak in a flow histogram of normal cells should have the width of only a single channel (all the particles should have the same fluorescence intensity and should appear in the same channel). In practice, because staining and illumination conditions will not be exactly uniform, the fluorescence intensities of normal nuclei stained with propidium iodide have a certain range of values. One of the ways in which otherwise quite civilized flow cytometrists compete with each other is by bragging about the small CVs on the peaks of their DNA histo-

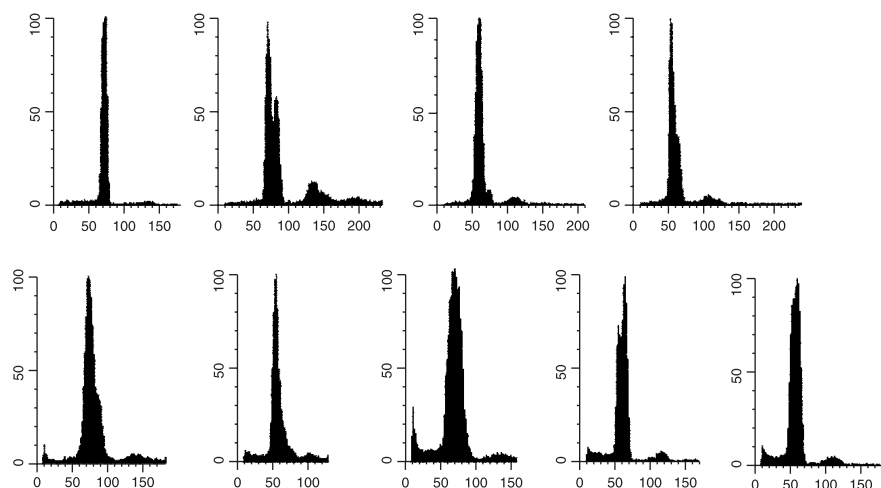


Fig. 8.2. Compared with the narrow peak in the normal histogram at the upper left, it can be seen that a single peak with a wide coefficient of variation (CV) or skewed profile may mask a near-diploid malignant cell line. In addition, an extra small peak at the 4C position may result from clumping of nuclei, cycling cells, or a true tetraploid abnormality. Data courtesy of Colm Hennessy.

grams (as a rule of thumb, $<3\%$ is good; $>8\%$ is not good). A wide CV might result from old and partially degraded material, from erratic flow due to a partially clogged flow orifice, from a fluctuating laser beam, from a sample that has been run too quickly (remember that widening of the core diameter within the sheath stream may lead to unequal illumination as particles stray from the center of the laser beam), from nuclei that have been unequally exposed to stain, or, finally, from abnormal cells with a DNA content quite close to that of the normal material. The sensitivity of the technique for detecting these near diploid abnormalities and thus for classifying tissue as euploid or aneuploid therefore depends on the cytometrist's ability to obtain narrow CVs in the normal controls.

Another problem concerning interpretation arises from the inconvenient fact that aneuploid tumors often have DNA content that is very close to double the amount found in normal cells. This amount is referred to as *4C* or *tetraploid*. If we stop and think, we can immediately see why this might lead to problems in flow analysis (see the small 4C peaks in Fig. 8.2). First of all, perfectly normal cells

with the 4C amount of DNA appear at certain phases in the cell cycle (just before cell division); therefore, if normal dividing cells are present, a significant number of particles may have double the 2C amount of DNA and will therefore appear in a peak at the tetraploid position. Second, remember that the flow cytometer is poor in its ability to distinguish large particles from clumped particles. It is not surprising, then, that the cytometer is, in the same way, inadequate at distinguishing a nucleus with double the normal amount of DNA from two normal nuclei clumped together. Scientists and clinicians usually resort to adopting some threshold value for classification purposes. For example, a sample with a 4C peak may be considered aneuploid only if the tetraploid peak contains more than 10% of the total number of nuclei counted; otherwise it will be considered normal on the assumption that about 10% of normal cells may appear in the tetraploid position owing to clumping and/or mitosis.

CELL CYCLE ANALYSIS

As mentioned above, normal cells will have more DNA than the 2C amount appropriate to their species at times when they are preparing for cell division. The cell cycle has been divided into phases (Fig. 8.3). Cells designated as being in the G₀ phase are not cycling at all; cells in G₁ are either just recovering from division or preparing for the initiation of another cycle; cells are said to be in S phase when they are actually in the process of synthesizing new DNA; cells in the G₂ phase are those that have finished DNA synthesis and therefore possess double the normal amount of DNA; and cells in M phase are in mitosis, undergoing the chromosome condensation and organization that occur immediately before cytokinesis (resulting in the production of two daughter cells, each with the 2C amount of DNA). A DNA flow histogram provides a snapshot of the proportion of different kinds of nuclei present at a particular moment. If we look at the DNA content of cells that are cycling (not resting), we will find some nuclei with the 2C amount of DNA (either G₀ or G₁ cells), some nuclei with the 4C amount of DNA (G₂ or M cells), and some nuclei with different amounts of DNA that span the range between these 2C and 4C populations (Fig. 8.4). A theoretical histogram distribution would look like Figure 8.5. Figure 8.6 shows an example of

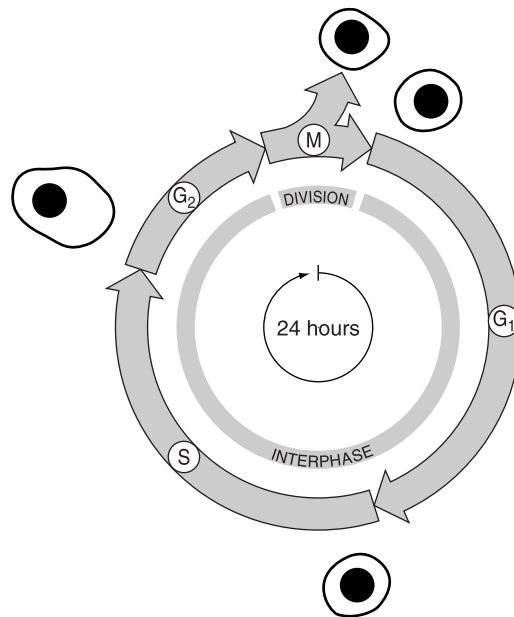


Fig. 8.3. The four successive phases of a typical mammalian cell cycle. From Alberts et al. (1989).

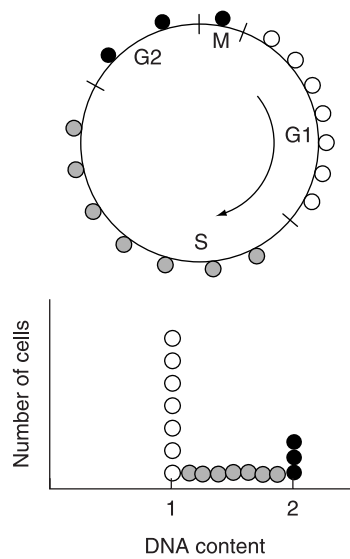


Fig. 8.4. Schematic illustration of the generation of a DNA distribution from a cycling population of cells. From Gray et al. (1990).

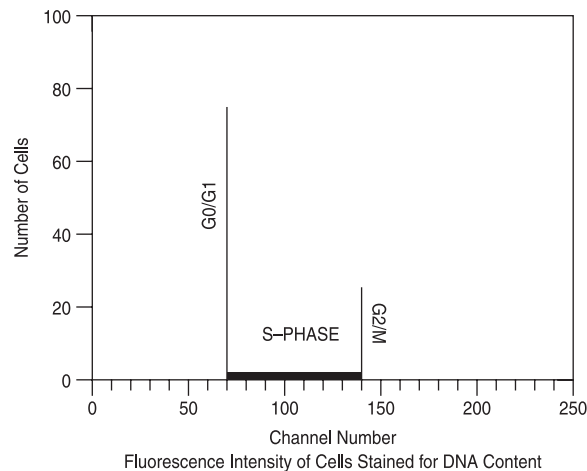


Fig. 8.5. The theoretical histogram generated from the sampling of a population of cycling cells.

DNA flow histograms that result from the propidium iodide staining of cells taken from a culture before and after they have been stimulated to divide.

The traditional method for analyzing cell division involves measuring the amount of DNA being synthesized in a culture by counting the radioactivity incorporated into DNA when the dividing cells are given a 6 h pulse with tritiated thymidine. The DNA histogram resulting from flow cytometric analysis offers an alternative to this technique. By dividing the histogram up with four markers, we can delineate nuclei with the 2C amount of DNA, those with the 4C amount of DNA, and those with amounts of DNA between the two delineated regions and therefore caught in the process of synthesizing DNA. The nuclei making DNA and showing up between the two peak regions should in some way correlate with the values obtained for DNA synthesis based on the uptake of tritiated thymidine. The values are not directly convertible one to the other: The radioactive method reflects the *total amount* of DNA being synthesized and will give higher values when more cells are present, whereas the flow method measures the *proportion* of cells that are in the process of making DNA and will not be affected by increases in the total number of cells. In addition, the radioactive method will give higher values if there is a significant amount of DNA repair going on,

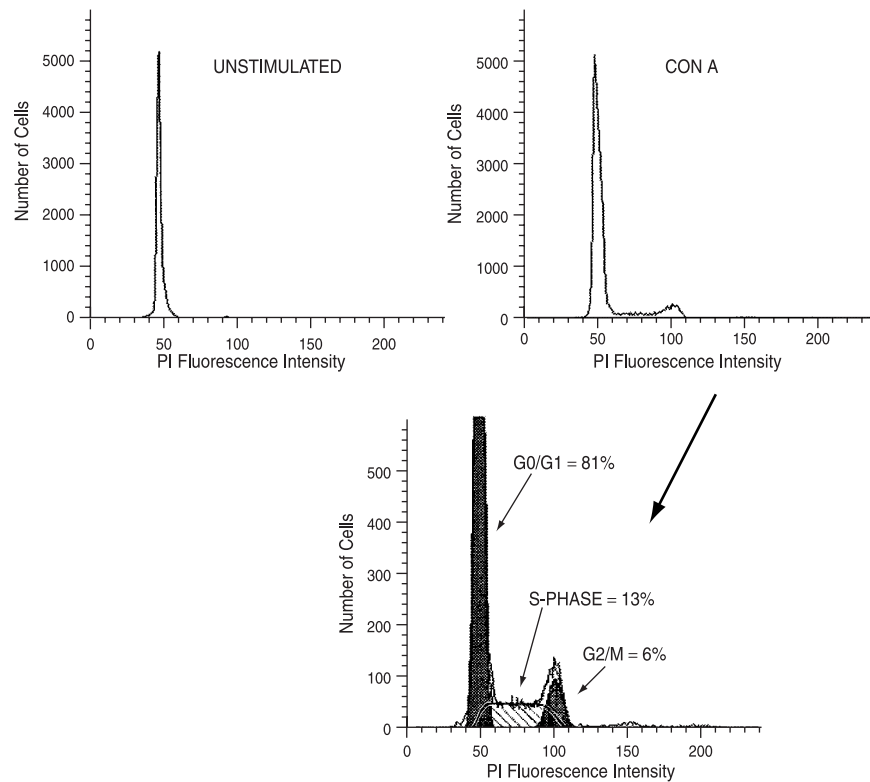


Fig. 8.6. DNA histograms from lymphocytes stimulated to divide.

whereas the flow method will give higher values if a proportion of cells are blocked in S phase. However, with these provisos, flow cytometry does offer a rapid and painless (nonradioactive) method for looking at cell proliferation.

Having agreed on the general principle that flow cytometry in conjunction with propidium iodide staining is an appropriate technology for analyzing cell proliferation, we have now to face the problem that the actual histogram has a certain width to the G₀/G₁ and to the G₂/M peaks and does not look like our theoretical distribution; we have to decide where to place those four markers mentioned above so as to delineate correctly the three regions (2C, 4C, and S phase). In a scenario that may by now be familiar, what seemed like a straightforward question turns out to have a less than

straightforward answer. Because the 2C and 4C peaks in a flow histogram have finite widths (remember the discussion about CV in the section on ploidy), it turns out to be rather difficult to decide where the 2C (or G0/G1) peak ends and nuclei in S phase begin. Similarly, it is difficult to know exactly where the distribution from nuclei in S phase ends and the spread from nuclei in G2 or M (4C amount of DNA) begins. In fact, there is no unambiguously correct point to place markers separating these three regions: The regions overlap at their extremes as a result of the inevitable nonuniformity of staining and illumination. The question therefore becomes not where to place the markers delineating the three cell cycle regions, but how many of the nuclei lurking under the normal spread of the 2C and 4C regions of the histogram are actually in S phase. Enter the mathematicians.

Algorithms based on sets of assumptions about the kinetics of cell division and the resulting shape of cell cycle histograms can be used to derive formulae for separating the contribution to the fluorescence distribution from our three separate cell cycle components. The algorithms range from the simple to the complex. They all seem to work reasonably well (that is, they all give similar and intuitively appropriate answers) when cell populations are well behaved. However, they all reflect the intrinsic limitations of using simplistic mathematical models for complex biological systems when cell populations grow too rapidly, are blocked in the cycle, or are otherwise perturbed. Bearing these limitations in mind, we can now look at four of the models used.

Figure 8.7 shows a DNA histogram derived from the propidium iodide staining of cells from a dividing culture. The simplest method for analyzing this histogram is the so-called *peak reflect method* whereby the shape of the G0/G1 peak is assumed to be symmetrically distributed around the mode. Given this assumption, the width of the peak, from the mode to the left (low fluorescence) edge is simply copied to the right (high fluorescence) edge; the same thing is done in reverse with the G2/M peak. Then everything in the middle between these two delineated regions is considered to be the result of S-phase cells.

A slightly more complex method for estimating the proportion of S-phase cells is called the *rectangular approximation method*. This method assumes that cells progress regularly through S phase and therefore that the proportion of cells at any given stage of DNA

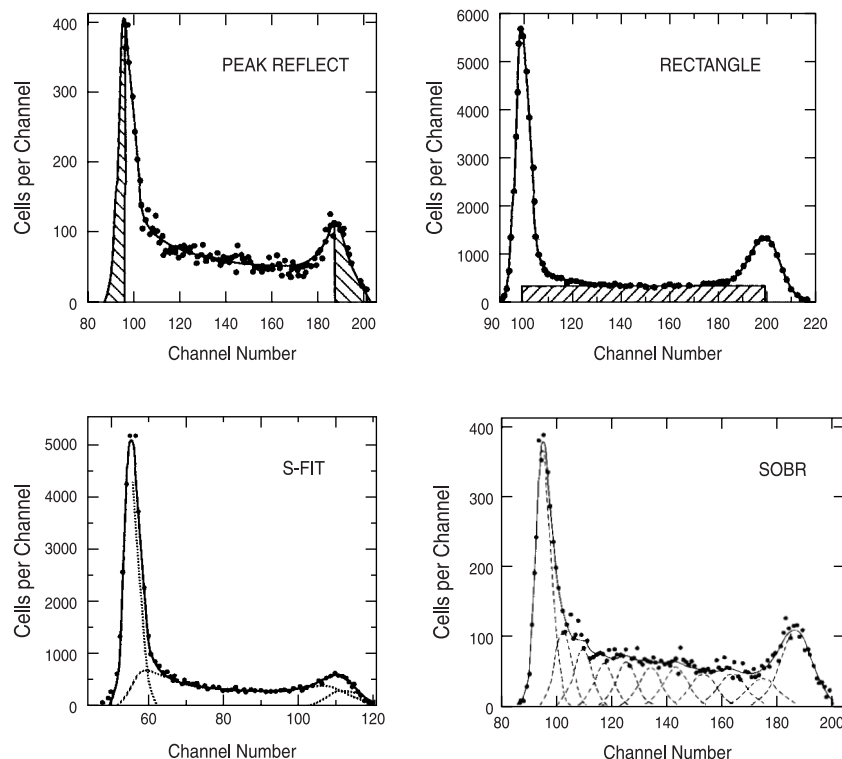


Fig. 8.7. Different mathematical algorithms for determining the contribution of S-phase nuclei to a DNA flow histogram. Upper left and lower right from Dean (1987); upper right and lower left from Dean (1985).

synthesis is constant. When this method is used, the average number of cells in the middle region of the DNA histogram is evaluated, and the height of this region is then extrapolated in both directions, toward the 2C peak and toward the 4C peak. The rectangle derived from this evaluation is then ascribed to S-phase cells, and all the other cells are considered either G₀/G₁ or G₂/M depending on whether they have higher or lower fluorescence than the middle point of the distribution.

The so-called S-FIT method and the sum-of-broadened-rectangles (SOBR) method both use more sophisticated mathematical assumptions to model the shape of the S-phase region of the histogram. A polynomial equation (S-FIT) or a series of broadened Gaussian dis-

tributions (SOBR) is derived that best fits the S-phase region of the histogram; then this derived shape is extrapolated toward the 2C and 4C peaks to estimate the contribution of S-phase cells within these regions.

One of the essential problems in assigning cells in a flow histogram to certain stages of the cell cycle is that an aggregate or clump of two G0/G1 cells will have double the normal DNA content (remember our discussion of the problem in diagnosing tetraploid tumors) and will appear as if they are a single G2/M cell. One way of diagnosing a clumped sample is by looking for peaks at the 6C position (resulting from three nuclei together). If there are clumps of three cells, then, statistically, there will be even more clumps of two cells. DNA analysis software can estimate the doublet contribution to the tetraploid peak by using probability algorithms to extrapolate from the triplet peak at the 6C position. The software (Fig. 8.8, right hand plot) can then subtract out this doublet contribution and give a measure of the “true” number of G2/M cells.

As well as software-based aggregate subtraction, so-called pulse processing (or, more recently, digital) electronics can give us help in this task. In general, particles (cells or nuclei) give out signals that

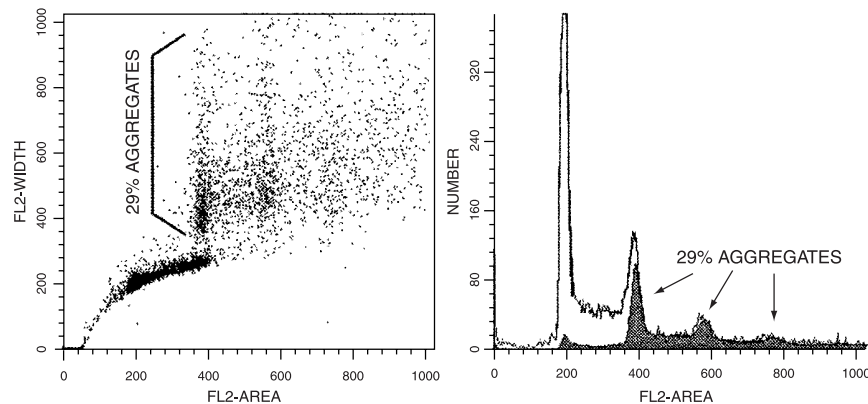


Fig. 8.8. By looking at the peak at the 6C and 8C positions (aggregates of three and four cells), software algorithms use this information to estimate the contribution of clumps of two cells to the peak at the G2/M (4C) position. The graph at the right indicates the software estimation of these aggregated cells. The graph at the left indicates where these cells fall on a plot of signal area versus signal width.

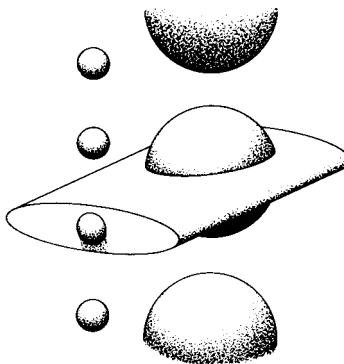


Fig. 8.9. The time that a fluorescence signal lasts (the signal width) depends primarily on the size of the laser beam (if the cell is smaller than the beam) or primarily on the size of the cell (if the cell is larger than the beam). From Peeters et al. (1989).

last, in time, just as long as it takes for the entire particle to move through the laser beam. What this means is that particles with diameters smaller than the laser beam all give out signals that last approximately the same length of time (dependent primarily on the laser beam width in the direction of flow and on the stream velocity); this is a traditional method of flow analysis. However, it is apparent that larger particles (or small particles in a very narrow laser beam) will give out signals whose time profiles are related primarily to their own diameter (Fig. 8.9). Pulse processing or digital electronics involves analysis of the full profile of the fluorescence signal from a particle. This includes a measure of the signal's width (the time it takes for the cell to pass through the laser beam), its height (the maximum fluorescence during this passage), and its area (the total fluorescence emitted during this passage) (Fig. 8.10). One large (G2/M) nucleus will pass through a narrow laser beam more quickly than will two smaller aggregated nuclei of the same total DNA content; however, the G2/M nucleus will have greater fluorescence intensity when it is centered in the beam (assuming a narrow laser beam relative to the diameter of two cells). Therefore, the signal area is used as the DNA parameter in cell cycle analysis because it is most closely proportional to total DNA content of a cell, but the width and height characteristics of the resulting fluorescence signal can be stored as extra parameters and can be used to distinguish clumps

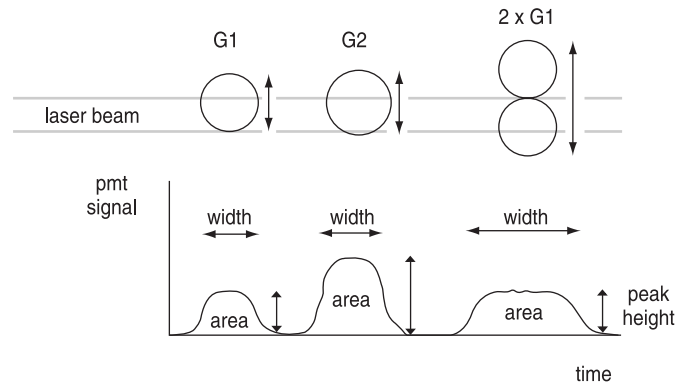


Fig. 8.10. Signal width, area, and height characteristics of light pulses as G1, G2, and two clumped G1 cells move through a narrow laser beam. Modified from Michael Ormerod.

from single G2/M particles. This can help considerably in the interpretation of DNA histograms (Fig. 8.11).

Software algorithms for estimating the proportion of S-phase cells are approximations. They can be refined mathematically in the hope of better approaching the biological truth. A mathematical model will always, however, have trouble coping with a biological situation that is disturbed or contains mixed populations behaving in erratic

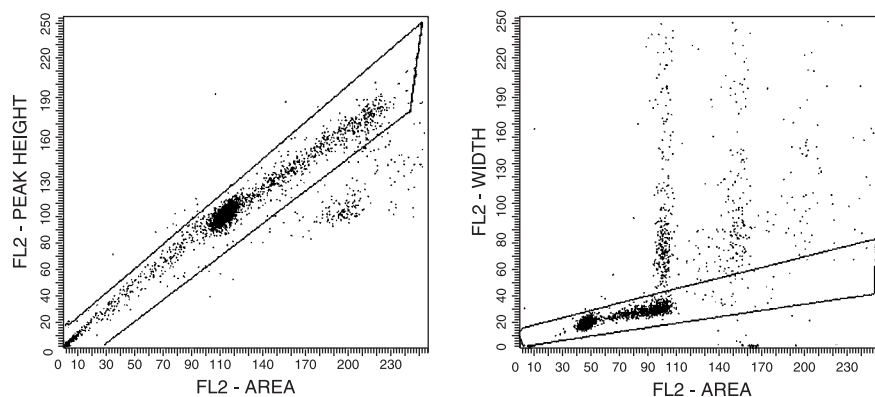


Fig. 8.11. Single cells (shown in the gates) can be distinguished from aggregates because single cells have lower signal widths and greater signal heights relative to their signal areas. The left plot is of data acquired on a Beckman Coulter cytometer; data in the right plot were acquired on a Becton Dickinson cytometer.

ways. Flow cytometry does, however, offer a more direct way to measure DNA synthesis. Bromodeoxyuridine (sometimes abbreviated BrdU or BUdR or BrdUdr) is a thymidine analog. If cells are pulsed with BrdU, it will be incorporated into the cell's DNA in the place of thymidine. Fluorescein-conjugated monoclonal antibodies with specificity for BrdU are available so that cells that have been pulsed with BrdU for a short period of time (about 30 min) can then be treated to partially denature their DNA, exposing the BrdU within the double helix so that it can be stained with the anti-BrdU antibody. Any cells that have incorporated BrdU during the pulse will then stain fluorescein positive.

The clever part of this technique is that the denatured DNA can be stained with propidium iodide at the same time. The resulting two-color contour plots look like those in Figure 8.12. The red fluorescence axis shows the propidium iodide distribution (proportional to DNA content) with which we have grown familiar; the green fluorescence axis shows which of these nuclei have actually incorporated BrdU during the pulse. As might be expected, the cells in the middle region of the propidium iodide distribution have all incorporated BrdU; but a proportion of the cells at either end of the propidium iodide distribution have also done so. This method, while somewhat

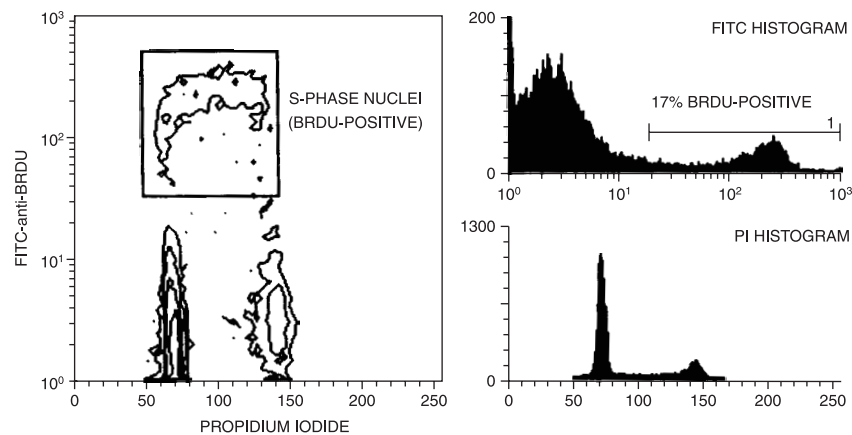


Fig. 8.12. Fluorescein (FITC) histogram, propidium iodide (PI) histogram, and dual-color correlated contour plot of human keratinocytes cultured for 4 days, pulsed with BrdU, and then stained with FITC-anti-BrdU and PI. Data courtesy of Malcolm Reed.

time consuming and a bit tricky technically, does allow a flow cytometrist to quantify the proportion of cells in S phase in a way that cannot be done accurately with simple propidium iodide staining. The bromodeoxyuridine method is, in fact, more comparable than simple propidium iodide staining to the traditional method of measuring cell division by assaying the incorporation of tritiated thymidine. It also provides the ability to distinguish cells that may be blocked in S phase from those that are actually incorporating nucleotides.

BrdU staining has also been used to provide information about the kinetics of cell cycles. If we consider cells pulsed for a short period of time with a small amount of BrdU and then killed immediately and stained with both propidium iodide and with a fluorescein-anti-BrdU monoclonal antibody, the fluorescein-antibody should stain equally all cells in S phase (stretching from the 2C peak to the 4C peak). If, however, we wait for some time before killing the cells (and if the BrdU has been used up quickly), then the cells that have incorporated the BrdU (that is, all the cells that were in S phase at the time of the pulse) will have synthesized more DNA, and some of those cells will have progressed into the G2 or M phase of the cell cycle (or indeed cycled back to G1). In addition, some new cells will have started to make DNA after the BrdU had been used up, and these cells will now be in S phase but will have DNA that does not contain BrdU. The contour plots for these cases look like those in Figure 8.13. We can estimate the rate of movement of the BrdU-containing cells through S phase and into the G2 peak by assuming that they are evenly distributed throughout S phase at the time of pulsing and then sampling and staining the cells at one subsequent time. The rate of increase in propidium iodide intensity of the fluorescein-positive nuclei is equivalent to their rate of DNA synthesis and provides us with information about the cycle time of the actively dividing cells. Moreover, the cycle time of the fluorescein-positive cells, in conjunction with the proportion of cells in S phase, can be used to estimate the doubling time of a population of cells.

Given the possible ambiguities in attempting to correlate clinical prognosis with aneuploidy and given the knowledge that malignant cells typically have “out-of-control” or unregulated proliferation, considerable work has been done in an attempt to use flow cytometry to correlate the percentage of cells in S phase with clinical prognosis in malignant disease. Although flow cytometrists tend to have reser-

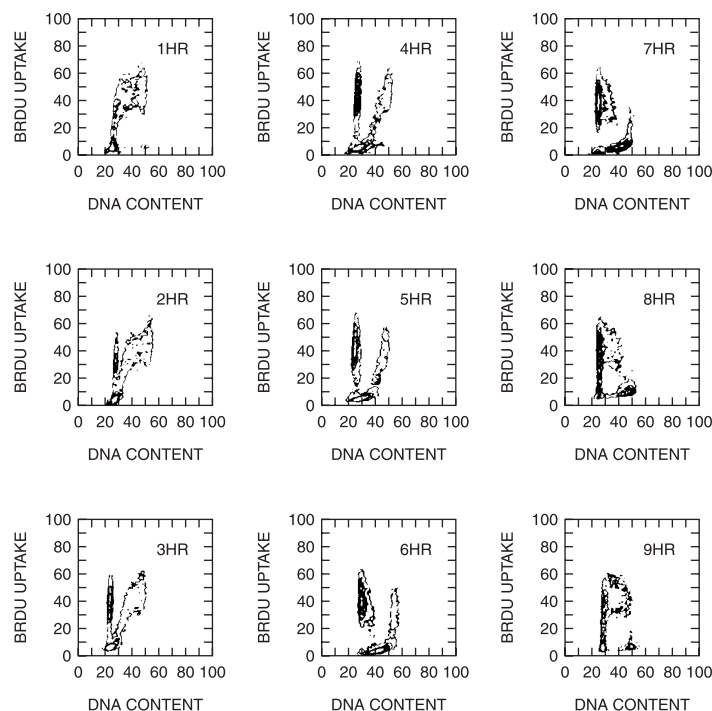


Fig. 8.13. Dual-color distribution of BrdU versus DNA content for Chinese hamster cells pulsed with BrdU and then sampled hourly. From McNally and Wilson (1990).

variations about the reproducibility of S-phase determinations from mathematical modeling of a propidium iodide fluorescence histogram (for the reasons mentioned above), many reports have been published in the medical literature showing useful correlation of the proportion of cells in S phase (the “S-phase fraction”) with poor clinical prognosis (see Chapter 10).

TWO-COLOR ANALYSIS FOR DNA AND ANOTHER PARAMETER

Having described the analysis of cells for total DNA content (with propidium iodide) and newly synthesized DNA (with BrdU), we may now go on to consider some other techniques for exploiting the multi-

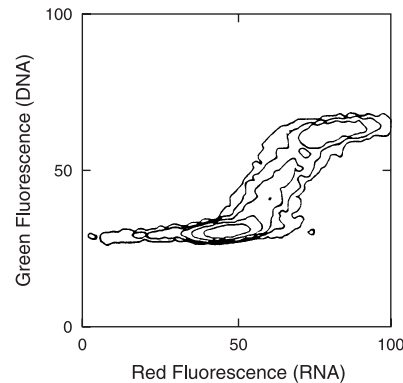


Fig. 8.14. DNA (green fluorescence) versus RNA (red fluorescence) content of human leukemic cells stained with acridine orange. From Darzynkiewicz and Traganos (1990).

parameter potential of the flow cytometer with the dual staining of cells for both DNA and some other parameter. Zbigniew Darzynkiewicz and coworkers in New York have used acridine orange with great success to look at the DNA and RNA contents of cycling cells. Because acridine orange fluoresces red when it binds to RNA and green when it binds to DNA, cells can be examined simultaneously for both constituents. Dual analysis of this type has given us increased information about the progression of cells through the cell cycle. Specifically, it can be seen quite clearly from plots like that in Figure 8.14 that some of the cells with the 2C amount of DNA (G0 or G1) contain increased levels of RNA. The synthesis of RNA appears to be an early event in the entrance of a cell into the division cycle. After this initial increase in RNA content, cells synthesize more RNA as they begin to synthesize DNA (S phase). At mitosis, they have increased levels of both RNA and DNA compared with resting cells. Analysis of the acridine orange staining of cycling cells has led, in particular, to improved ability to analyze early events in the division cycle.

This acridine orange technique has been taken one step further by Darzynkiewicz's group. Because acridine orange fluoresces red when bound to single-stranded nucleic acid, but green with double-stranded nucleic acid, acridine orange can be used under mildly denaturing

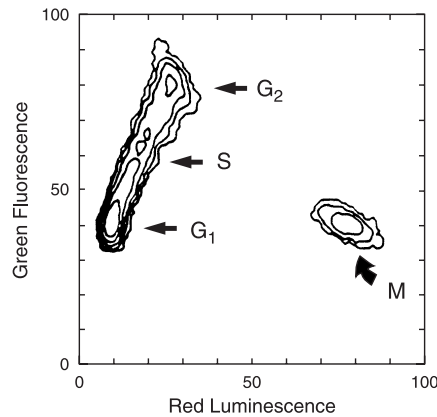


Fig. 8.15. L1210 cells treated with RNase and acid and then stained with acridine orange reveal that DNA in mitotic cells is extensively unwound and exhibits increased red and decreased green fluorescence relative to DNA from cells in other phases of the cell cycle. From Darzynkiewicz (1990).

conditions (and in the presence of RNase) to distinguish easily denatured from more resistant forms of DNA. Once RNase has been used to remove the RNA, mild denaturation can be used to cause unwinding of the helical forms of DNA that are loosely packed (e.g., in cells that are in the process of DNA synthesis); it will not affect DNA that is tightly condensed (e.g., in resting cells). Figure 8.15 indicates the kind of contour plots that can be obtained from this type of procedure.

Dual staining of cells can also be used to look at DNA and protein markers simultaneously. Using methods similar to those described in the previous chapter for looking at cytoplasmic proteins simultaneously with surface membrane proteins, cells can be stained for surface proteins, then fixed and permeabilized, and then stained for DNA. Figure 8.16 shows the way that this type of technique can be used to permit the cell cycle analysis of subpopulations of cells independently. In this particular example, it can be seen that, after treatment with the mitogen phytohemagglutinin, it is the CD8-positive cells more than the CD8-negative cells that have been induced to enter S phase.

Similar techniques can be used to stain cells for both DNA and internal (cytoplasmic or nuclear) markers. By fixing the cells, then

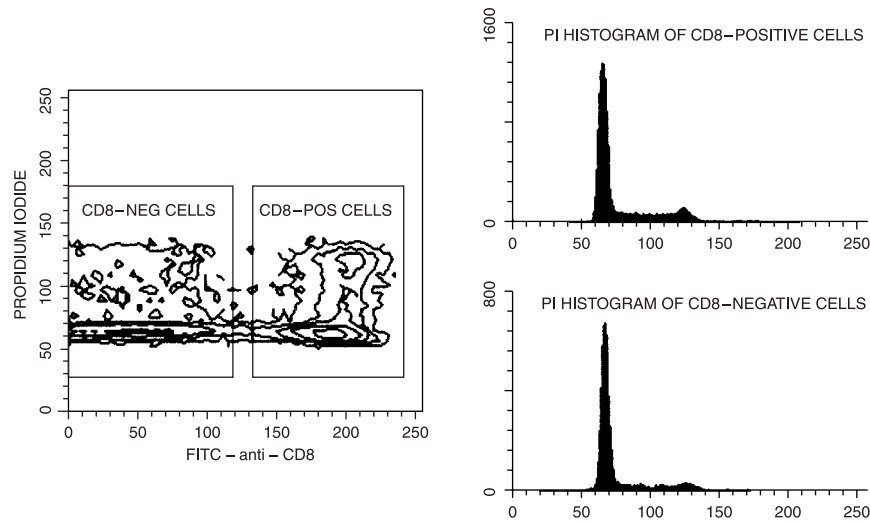


Fig. 8.16. Cell cycle analysis of CD8-positive and CD8-negative cells. Lymphocytes were cultured with phytohemagglutinin, stained with FITC-anti-CD8 monoclonal antibody, treated with saponin to permeabilize the outer membrane, and then stained with propidium iodide (PI) and RNase. Cells provided by Ian Brotherick.

staining for cytoplasmic markers like cytokeratin (as in the example of staining tumor cells for cytokeratin and estrogen receptors in the previous chapter, Fig. 7.3), and then staining with propidium iodide, classes of cells can be selected for ploidy analysis. In this way, for example, breast tumor cells (which are likely to be of epithelial origin) can be gated in a mixed population from a tumor. Then the cells gated for fluorescein-anti-cytokeratin positivity can be further analyzed to see if any of these nuclei are aneuploid. By this technique, minor populations of tumor cells within a heterogeneous population may be selected and their ploidy determined with more sensitivity.

The Darzynkiewicz laboratory has continued, more recently, to use flow cytometry in creative ways to contribute to our knowledge of the regulation of cell division. By staining for DNA as well as cell cycle-regulatory proteins (the cyclins), the presence of these proteins can be associated with certain stages of the cell cycle. Figure 8.17 shows an example of data derived from a protocol staining cells for DNA content along with antibodies against the nuclear proteins H3-P (H3 is a histone, which, when phosphorylated, is associated

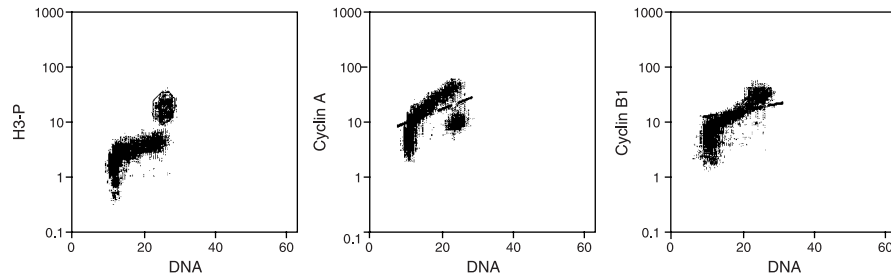


Fig. 8.17. The use of stains for the intracellular proteins H3-P, cyclin A, and cyclin B1 in conjunction with propidium iodide to distinguish cells with the same DNA content but at different stages of the cell cycle. From Juan et al (1998).

with chromosomal condensation), cyclin A (a cycle-regulatory protein that begins to accumulate at mid-S phase, reaches maximal concentration at the end of G₂, and is degraded early in mitosis), and cyclin B1 (which begins to accumulate at the beginning of G₂ and is maximal at the beginning of mitosis, declining at the cell's entry into anaphase). H3-P can dichotomize 4C cells into those that are in the G₂ stage of the cycle and those that are actually mitotic. Similarly, the cyclins can additionally separate cells at different stages of mitosis. Figure 8.18 indicates a summary of the time course for expression of cyclins D, E, A, and B.

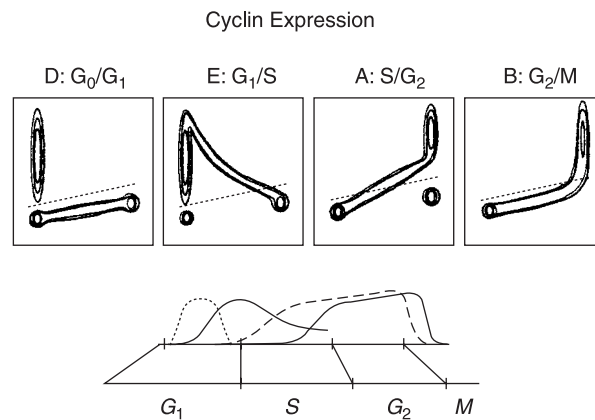


Fig. 8.18. A summary of the time course of expression of cyclins D, E, A, and B through the cell cycle. DNA is plotted on the x-axis of the contour plots and cyclin expression on the y-axis. Courtesy of James Jacobberger.

Some particular issues arise when combining DNA staining with protein staining; these issues are related to those discussed in the previous chapter on combining the staining of intracellular with extracellular proteins. In both cases, the fixation procedure, while maintaining the integrity of the cells after permeabilization, can also affect the stainability of the molecules in question. In the case of DNA staining, fixation of cells will cross-link histones on the chromosomes, thus limiting the access of DNA-specific fluorochromes to their binding sites. In practice, this means that cells fixed with formaldehyde will maintain their cytoplasmic integrity well but will show decreased propidium iodide fluorescence and wider CVs than will cells fixed with, for example, ethanol. The solution is, once again, a compromise. Short fixation with low concentrations of formaldehyde followed by detergent treatment works well to permit maintenance of cytoplasmic proteins along with fairly good DNA profiles. Exact procedures need to be individualized to the proteins and cells in question.

CHROMOSOMES

Until now, the particles flowing through our flow cytometer have been cells or nuclei. Other types of particles are, of course, possible. One of the best examples of the successful application of flow cytometry to noncellular systems has been in the analysis of chromosomes. In this case, the particles flowing through the system are individual chromosomes that are released from cells that have been arrested in metaphase (much the same conditions as those that are used to prepare chromosomes for analysis in metaphase spreads under the microscope). The released chromosomes are stained with a DNA stain (like propidium iodide) and then sent through the flow cytometer. The resulting histograms of fluorescence intensity reveal peaks whose positions along the x-axis are proportional to the amount of DNA in the chromosome and whose areas are proportional to the number of chromosomes with that particular DNA content (Fig. 8.19). Histograms of this type are called *flow karyotypes*, by analogy with the microscope karyotypes derived from conventional genetic analysis.

Figure 8.20 shows examples of flow karyotypes from different

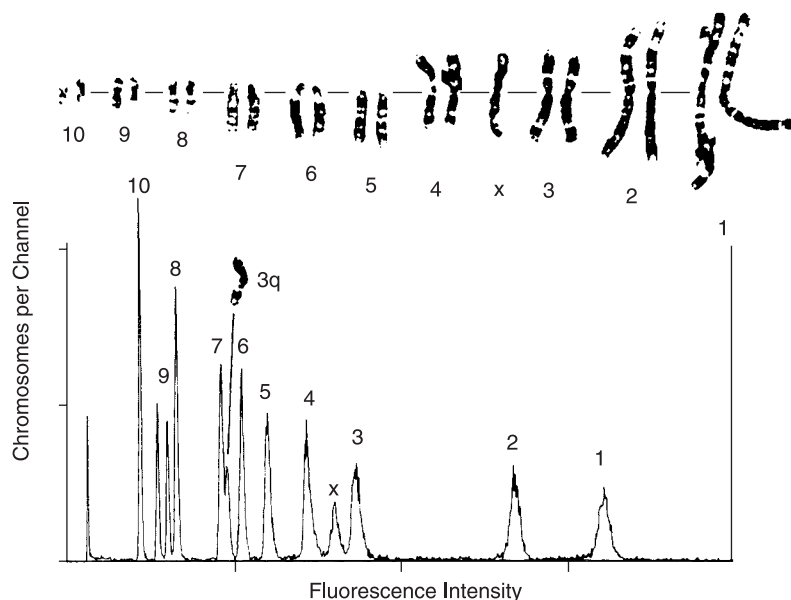


Fig. 8.19. A flow karyotype (fluorescence histogram) of Chinese hamster chromosomes stained with propidium iodide (PI). The G-banded chromosomes from this particular aneuploid cell line are included for comparison with the histogram peaks. From Cram et al. (1988).

species. While some species with small numbers of chromosomes reveal relatively simple histogram patterns, the 23 pairs of chromosomes in the human lead to a rather complex pattern. It is apparent that, although 23 pairs of chromosomes are readily distinguished under the microscope by a combination of size, centromere position, and banding patterns, many of these pairs have similar total DNA content and are not distinguishable in a flow histogram. We can obtain considerable help by using Hoechst 33258 and chromomycin A3 in a dual staining system: Hoechst 33258 stains adenine- and thymine-rich regions of DNA preferentially, and chromomycin A3 is specific for regions rich in the guanine and cytosine base pairs. By using this dual system, we find that some chromosomes with closely similar total DNA content have differing base pair ratios. Compare particularly the positions of chromosomes 13–16 in the one-dimensional histograms (Fig. 8.20A) with these same chromosomes (now separable) in the contour plot (Fig. 8.21). Unfortunately, chromosomes 9–12 are not distinguishable with either system.

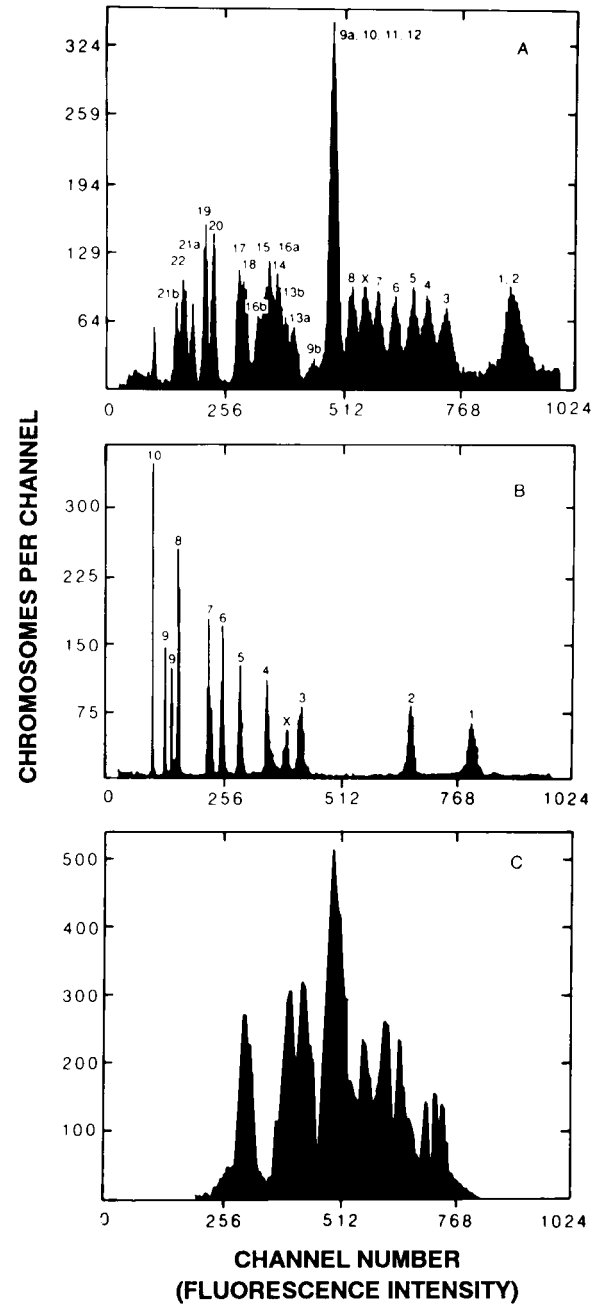


Fig. 8.20. Flow karyotypes from human chromosomes (A), hamster chromosomes (B), and mouse chromosomes (C). From Gray and Cram (1990).

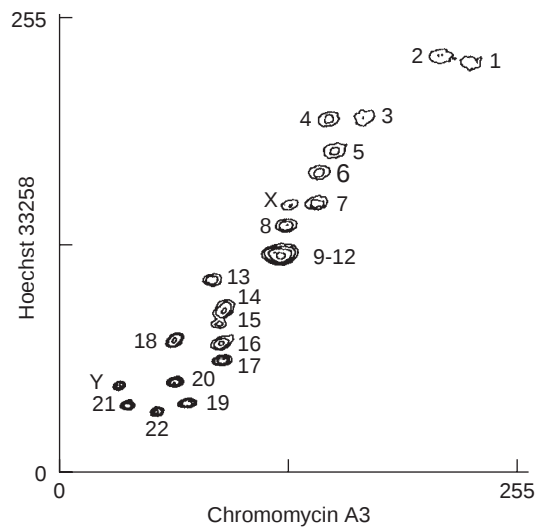


Fig. 8.21. A bivariate flow karyotype for human chromosomes stained with Hoechst 33258 and chromomycin A3. From Gray and Cram (1990).

There has been much discussion about the potential utility of flow cytometry of chromosomes for clinical diagnosis. As regards its sensitivity, this technique appears to stand somewhere between the technique of flow analysis of whole cells for DNA content and that of microscope analysis of banded chromosomes. It may be a useful intellectual exercise for readers to ask themselves which technique or techniques would be most appropriate for detecting the following types of chromosome abnormalities: (1) tetraploidy, where the normal chromosome content of cells is exactly doubled because of failure of cytokinesis after mitosis; (2) an inversion in an arm of one particular chromosome; and (3) trisomy (the existence of cells with three instead of two) of one of the small chromosomes. In addition to these limitations, the use of flow cytometry to look for abnormal chromosomes has been confounded by the fact that several human chromosomes are highly polymorphic, and flow karyotypes, therefore, vary considerably among normal individuals.

APOPTOSIS

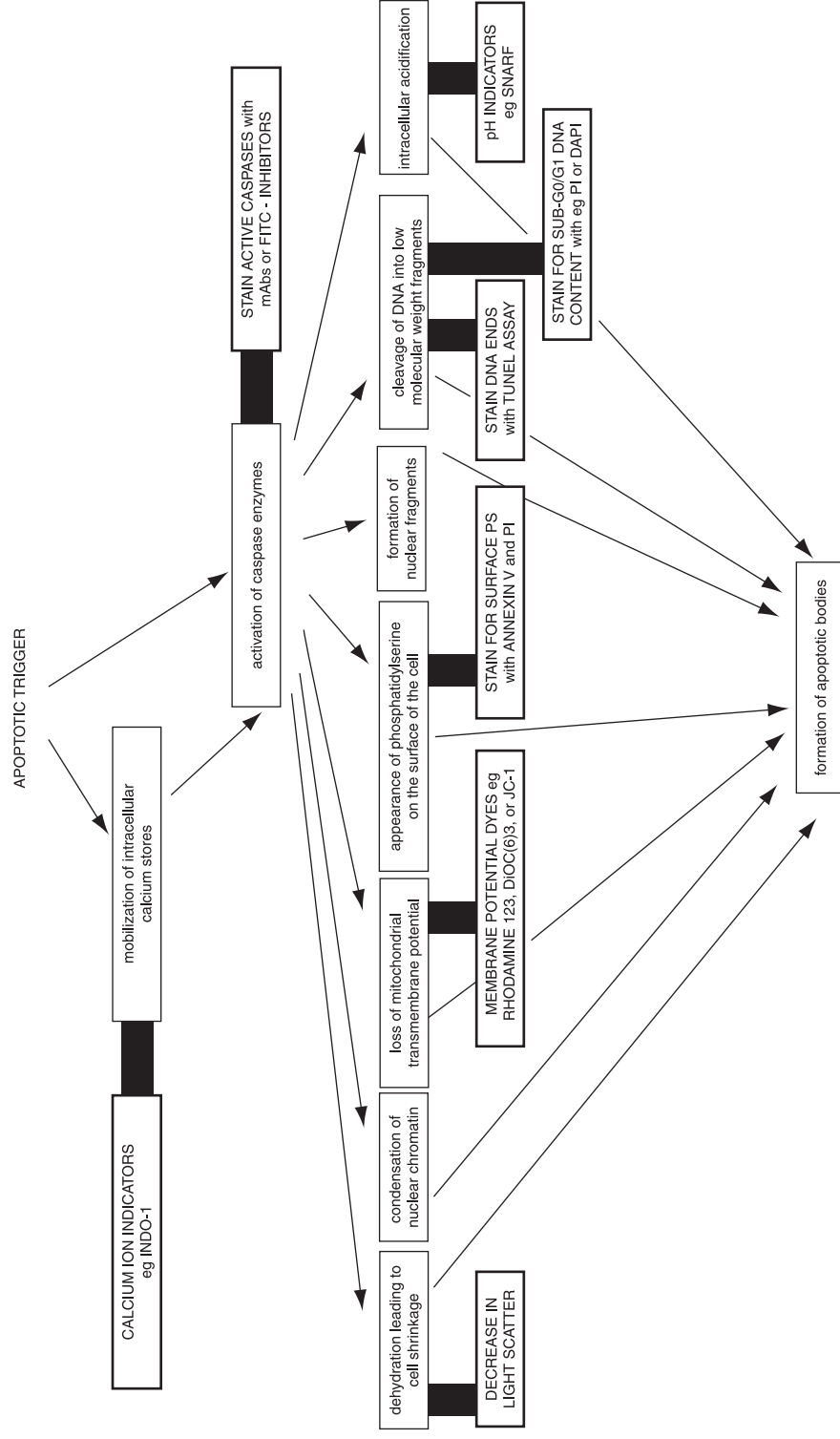
We can close this chapter with discussion of two “end of life” applications—apoptosis and necrosis. Although a naïve observer

might see death as an unfortunate (and scientifically boring) endpoint, recent work indicates that cell death is neither boring nor necessarily unfortunate. Cells often die by an active process that is an important part of the maintenance of organismal homeostasis. This process is called *apoptosis*. Apoptosis, or programmed death, can, for example, prevent the survival of potentially malignant cells with damaged DNA.

Cells that are undergoing apoptosis progress through a series of events. Many of the events that we measure in association with apoptosis, however, may be overlapping in time and therefore may not be a “pathway” so much as symptoms of the underlying process. Some of the events are not obligatory and may differ depending on the nature of the apoptotic trigger and the cell type. In any case, several of these apoptotic-associated events can be analyzed by flow cytometry (Fig. 8.22). One of the events is the flipping and stabilization of phosphatidylserine from the inner surface of the cytoplasmic membrane to the outer surface. On the outer surface, phosphatidylserine appears to identify cells as targets for phagocytosis. Because annexin V binds to phosphatidylserine, staining of intact cells with fluorochrome-conjugated annexin V will detect cells that are in early stages of apoptosis.

A two-color dot plot (Fig. 8.23) of cells stained with propidium iodide and annexin V–FITC will indicate cells in three of the four quadrants. Unstained cells are alive and well and are the double negatives; they neither express phosphatidylserine on their surface nor take up propidium iodide through leaky membranes. Cells that stain just with annexin V are apoptotic; they have begun to express phosphatidylserine on their surface, but have not yet gone through the process that leads to permeabilization of their cytoplasmic membrane. Cells that stain both with propidium iodide and annexin V are necrotic (that is, dead); they take up propidium iodide and also stain with annexin V. With a permeable cell, the flow cytometer cannot tell us whether the annexin V is on the outside of the membrane (because the cells have gone through apoptosis before membrane permeabilization) or on the inside of the membrane (because the cells have died by the necrotic pathway without apoptosis).

Another event associated with apoptosis is the fragmentation of DNA due to endonuclease activation. This fragmentation results in the appearance of increased numbers of “free ends” on the DNA molecules in the cell. By incubating fixed and permeabilized cells with



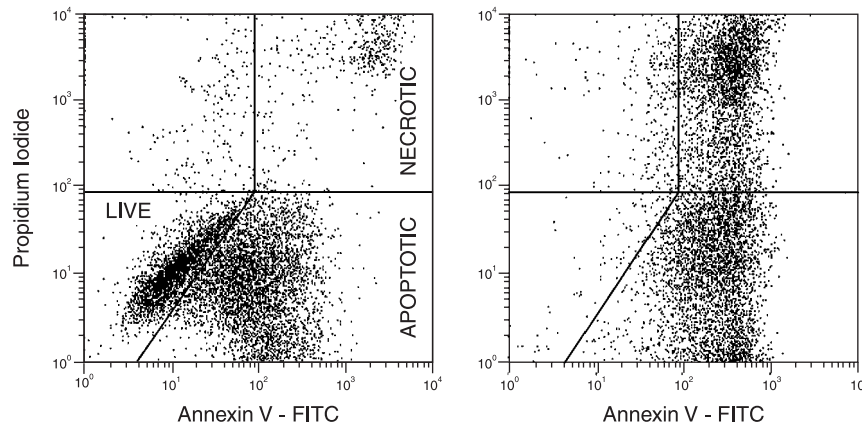


Fig. 8.23. Cells stained with propidium iodide for permeable membranes and with annexin V for phosphatidylserine can distinguish live (double negative), apoptotic (annexin V-positive, propidium iodide-negative), and necrotic (double positive) cells. This figure (with data from Robert Wagner) shows cultured bovine aortic endothelial cells; the left plot displays data from adherent cells, and the right plot displays data from the “floaters.”

the enzyme terminal deoxynucleotidyl transferase (TdT) and with fluorochrome-labeled nucleotides, cells with greater numbers of DNA fragments incorporate more of the fluorescent nucleotides onto their increased number of termini. The resulting increased cellular fluorescence is indicative of an apoptotic cell (Fig. 8.24, lower dot plots). Appearance of positive cells in this TdT flow assay correlates well with the classic gel electrophoresis assay for apoptosis, where “ladders” of small DNA fragments are indicators of endonuclease activation. In a desperate attempt to find an acronym, this flow assay for apoptosis has been called the TUNEL assay (for *Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labeling*).

Propidium iodide staining alone can be used to detect later stages of apoptosis (Fig. 8.24, upper histograms). When the DNA becomes extensively fragmented, the small fragments begin to be ejected from

◀ **Fig. 8.22.** A diagram of some of the events associated with apoptosis, showing related flow cytometric assays (outlined in bold boxes). Although it is generally agreed that calcium flux and caspase activation are early events following the trigger signal, the events following on from there may be essential steps in an ordered pathway or may be independent and merely symptomatic of apoptosis.

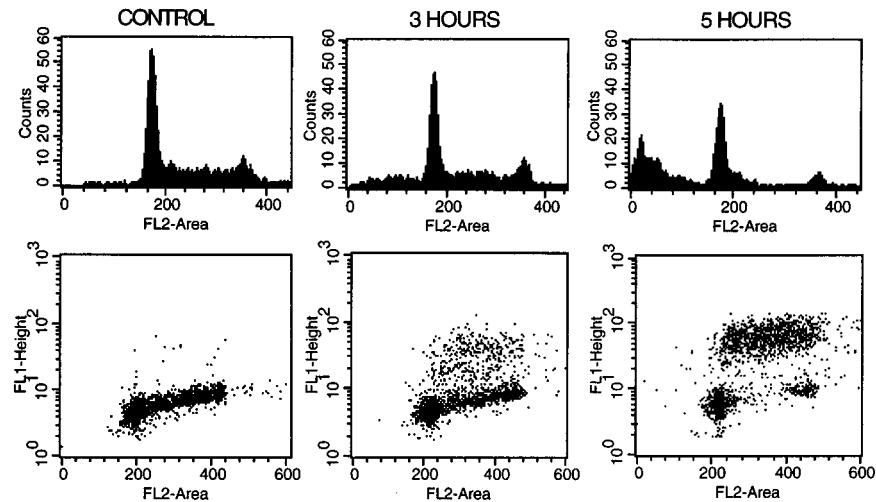


Fig. 8.24. Cells in late apoptosis have fragmented DNA. They can be visualized as cells with increased incorporation of fluorochrome-labeled nucleotides to the ends of the fragments in the flow cytometric TUNEL assay (lower dot plots, with FL1 as FITC-dUTP and FL2 as propidium iodide bound to DNA). Alternatively, they can be visualized as cells with less-than-normal (sub-G0/G1) DNA content (upper histograms of propidium iodide fluorescence). Data from etoposide-treated ML-1 cells courtesy of Mary Kay Brown and Alan Eastman.

the cells and/or can be washed from the cells during the staining protocol. In either case, the apoptotic cell at this stage will have significantly less DNA than a healthy, viable cell. This lowered DNA content can be detected as a “sub-G0/G1” peak in a simple, one-color DNA histogram. It needs to be mentioned that staining for the cells with sub-G0/G1 DNA content requires a protocol that encourages apoptotic cells to release their fragmented DNA. In contrast, the TUNEL assay uses fixation to ensure that the fragmented DNA is retained.

NECROSIS

Unlike apoptosis, which involves the scheduled and active coordination of metabolic processes, necrosis is a passive response to a toxic or injurious environment. Whereas in apoptosis the cell membrane remains intact until very late in the game, permeabilization of the cell membrane is an early event in necrosis. Because propidium iodide

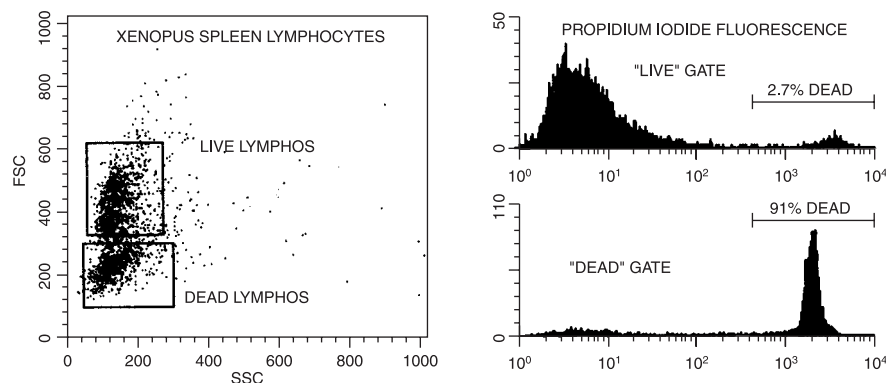


Fig. 8.25. The use of propidium iodide to monitor cell death. Dead lymphocytes have a less intense forward scatter signal than do live lymphocytes. Cultured *Xenopus* spleen lymphocytes courtesy of Jocelyn Ho and John Horton.

is excluded from entering cells by an intact plasma membrane and because it only fluoresces when intercalated between the bases of double-stranded nucleic acid, it will not fluoresce if it is added to a suspension of intact cells. The intact plasma membrane forms a barrier, keeping propidium iodide and nucleic acids apart. It is only when the outer membrane has been breached that the cells will emit red fluorescence. Propidium iodide (or other membrane-impermeant DNA fluorochromes) is therefore a stain (like trypan blue) that can be used to mark necrotic cells (on the reasonable but not necessarily valid assumption that cells with holes in their membranes large enough to allow the penetration of propidium iodide are actually dead according to other viability criteria and vice versa). By this method, cell viability can be monitored in the presence of various cytotoxic conditions (Fig. 8.25).

The only difficulty in using flow cytometry to monitor cell death is that, as mentioned in Chapter 3, dead cells have different scatter properties than living cells. In particular, because of their perforated outer membrane, they have a lower refractive index than living cells and therefore have forward scatter signals of lower intensity. For this reason, it is important not to use a gate or forward scatter threshold when analyzing a population for the proportion of dead and live cells. Any forward versus side scatter gate drawn around normal lymphocytes, for example, will always show most if not all of the cells

within that gate to have excluded propidium iodide no matter how many cells in the preparation are dead; this is simply because the dead cells drop out of the gate (Fig. 8.25). The lesson here (and one that needs repeating in reference to most flow analysis) is that gating is an analytic procedure that needs to be performed carefully and with explicit purpose. If we do not define our population of interest (by gating) with care, our results may be accurate answers to inappropriate questions (e.g., “what percentage of living lymphocytes are alive?”).

The use of membrane-impermeant DNA fluorochromes to mark dead cells has a more routine application in simply allowing the exclusion of dead cells from flow analysis. This is important because dead cells have the habit of staining nonspecifically when their broken membranes tend to trap monoclonal antibodies directed against surface antigens. Therefore a cell suspension including many dead cells may show high levels of nonspecific stain. By adding propidium iodide to cells just before analysis, the cells fluorescing red can be excluded from further analysis by gating on red negativity and only the living cells then examined for surface marker staining. Figure 8.26 shows the way that this technique can be used when looking at fluorescein staining for surface antigens on cultured mouse thymocytes.

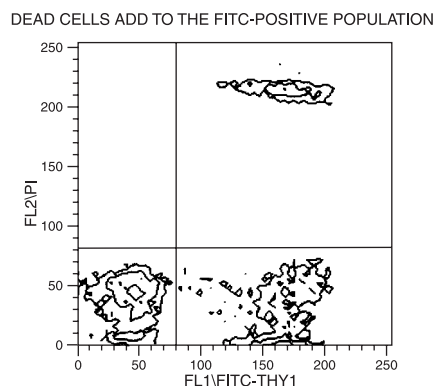


Fig. 8.26. The use of propidium iodide to exclude dead cells from analysis of a mouse spleen cell population for the expression of the Thy-1 surface antigen. The cells were stained with fluorescein for Thy-1 and then with propidium iodide to mark the dead cells. It can be seen that the dead cells (upper right quadrant) contribute to the fluorescein-positive population. Stained cells courtesy of Maxwell Holscher.

The use of propidium iodide or equivalent dyes to exclude dead cells from analysis is a procedure that is recommended as routine for any system staining for surface markers. It is particularly important in analysis of mixed populations where a high percentage of the cells may be dead.

It should, however, be remembered that fixed cells cannot be stained with propidium iodide for live/dead discrimination. Fixed cells are, in fact, all dead and will therefore all take up propidium iodide even if some were alive and some dead before fixation. Ethidium monoazide offers an alternative to propidium iodide if cells will be fixed before flow analysis. It is a dye that, like propidium iodide, only enters dead cells. It has, however, the added advantage of forming permanent cross-links with DNA when photoactivated. Therefore, cells can be stained for surface proteins, incubated with ethidium monoazide under a desk lamp, washed, and then fixed. At the time of acquisition of data on the flow cytometer, red fluorescence will mark the cells that were dead before fixation.

FURTHER READING

Chapters 13–16 and 24 in **Melamed et al.** and Chapters 11–13 in **Watson** are detailed reviews of the applications of flow cytometry to various aspects of cell cycle research.

Several chapters in **Ormerod** and several sections in the **Purdue Handbook**, in **Diamond and DeMaggio**, and in **Current Protocols in Cytometry** give good practical protocols. **Darzynkiewicz** (both the 1994 and 2001 editions) include many chapters with protocols and advice on both the simpler and the more complex methods in nucleic acid, apoptosis, and cell cycle analysis.

Good discussions of the mathematical algorithms for cell cycle analysis can be found in Chapter 6 of **Van Dilla et al.**, Chapter 23 of **Melamed et al.**, and in the multiauthor book *Techniques in Cell Cycle Analysis*, edited by Gray JW and Darzynkiewicz Z (1987), Humana Press, Clifton, NJ.

The “Cytometry of Cyclin Proteins” is reviewed by Darzynkiewicz Z, et al. (1996) in *Cytometry* 25:1–13.

Molecular biology and chromosome analysis are discussed in Chapters 25–28 of **Melamed et al.** A special issue (Volume 11, Number 1, 1990) of the journal *Cytometry*, although somewhat aged, is devoted to the subject of

analytical cytogenetics; it contains articles about work at the interface between theory and clinical practice (as well as some beautiful pictures).

A review article by Darzynkiewicz Z, et al. (1997) on “Cytometry in Cell Necrobiology: Analysis of Apoptosis and Accidental Cell Death (Necrosis)” appeared in *Cytometry* 27:1–20. Boehringer Mannheim produces a free, but substantial manual (now in its second edition) on methods related to the study of cell death.